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13. ABSTRACT ***(Maximum 200 Words)***

During this four-year funding and one-year no cost extension periods we have established a rat model of active specific immunotherapy against mutated epidermal growth factor receptor (EGF-RvIII) specifically expressed by breast carcinomas. The mutation is not expressed by normal breast tissues. The model system consists of rat mammary carcinomas expressing EGF-RvIII after cDNA transfer and EGF-RvIII vaccines, including recombinant EGF-RvIII protein, EGF-RvIII expressed in adenovirus vector and peptides mimicking EGF-RvIII. Expression of EGF-RvIII by the transfected rat mammary carcinoma cells was instable due to vector mutations. The vector was corrected but rat mammary carcinoma cells transfected with the vector did not stably express EGF-RvIII. Future studies will be aimed at obtaining stable transfectants. These will allow us to test the available vaccines for their tumor growth inhibiting properties.

We have also evaluated breast cancer patients' immune responses to EGF-RvIII. Most patients with EGF-RvIII-positive lesions had circulating antibodies, and a few patients also EGF-RvIII specific lymphoproliferative responses. These responses may be beneficial to the patients, and this issue will be addressed in future studies in a larger population of patients.

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INTRODUCTION

(adapted from the previous report)

Clinical trials of active immunotherapy in breast carcinoma patients have suffered from the use of vaccines that induce only humoral, but not cellular, immunity (1,2) or lack specificity (3). Preclinical and clinical studies with cancer vaccines have demonstrated a correlation between the induction of humoral or cellular immunity and tumor growth inhibition (4-12). Thus, tumor vaccines ideally should induce both humoral and cellular immunity and induced immunity should be specific for the tumor cells. The major goal of this study is to test tumor-specific vaccines against breast carcinoma in a relevant rat model. Mutated epidermal growth factor receptor (EGF-RvIII) is expressed by a high proportion of breast carcinoma tissues derived from various patients, but not several normal tissues tested (13; and our unpublished data described in the original proposal). EGF-RvIII is expressed both on the surface and in the cytoplasm of tumor cells (13), rendering it a target for both B and T cells. Furthermore, targeting of EGF-RvIII may exert direct anti-proliferative effects (14).

We have chosen a rat model of EGF-RvIII vaccines for the proposed studies because of the availability of cloned normal rat EGF-R (15) and MHC class I and II positive rat mammary carcinoma cells with either high or low metastatic capability (16,17). The most specific vaccine of EGF-RvIII consists of the minimal sequence, including the mutation, that elicits B- and/or T-cell responses. We have chosen peptides of EGF-RvIII for induction of T cells, analogous to studies performed successfully with peptide vaccines by other groups (18-24) and our collaborators (25-27) in various antigen systems. In addition, recombinant, extracellular EGF-RvIII protein and full-length EGF-RvIII expressed in adenovirus vector have been chosen for induction of protective humoral and cellular immunity, respectively.

In conclusion, EGF-RvIII is a unique target for active specific immunotherapy of breast carcinoma, based on its specificity, frequency of expression, potential for activating both B and T cells, and availability of an ideal animal model of active immunotherapy. The studies will provide the rationale for specific active immunotherapy of breast carcinoma patients. The results we will obtain with EGF-RvIII in the rat mammary carcinoma model may be applicable to other tumor systems, such as lung carcinomas and gliomas which also express EGF-RvIII (13,28).

BODY

This final report includes a 4-year funding period and a 1-year no-cost extension period. The final report has been adapted from the previous report and the progress made during the past year (no cost extension period) for tasks 1 and 10 is indicated in italics.

Task 1: Transfection of two rat mammary carcinoma cell lines with available EGF-RvIII cDNA. Characterization of the transfectants.

Rat mammary carcinoma transfectants MTLN3 were generated which expressed the 145 kDa rat EGF-RvIII protein by Western blot analysis of whole cell extract, but did not express the protein in membrane extract. The transfectants did not react with murine monoclonal antibody

(MAb) L8 directed to the mutated epitope on EGF-RvIII. Thus, the transfectants did express EGF-RvIII protein, but lacked expression of the mutated epitope. They were tumorigenic in syngeneic rats. We had attributed our failure to obtain rat mammary carcinoma cells stably expressing rat EGF-RvIII protein to mutational events that possibly had occurred in the transfectants in vivo. Therefore, in order to obtain stable transfectants, we performed four independent transfections of 115 million cells total using various methods of transfection. Forty-eight colonies were isolated that showed 40% or more of the cells within a colony expressing EGF-RvIII defined by MAb L8A4. However, all colonies lost EGF-RvIII expression after 3-4 months in culture. Furthermore, attempts to isolate EGF-RvIII cells by fluorescence-activated cell sorting were unsuccessful. We therefore sequenced the EGF-R PcDNA3 vector used for cell transfection. There were two mutations each in the extracellular and intracellular domain of the EGF-RvIII vector. These mutations must have occurred at some point during EGF-RvIII vector propagation in vitro as they were not found in the vector when it was originally constructed and sequenced.

The data summarized above have been described in detail in Fig. 2 of the July 1997 report, and in the 1998 and 1999 reports.

During year 4, we sequenced the normal (non-mutated) EGF-R vector and found the sequence to be identical with the published sequence in Gene Bank. This vector was then used to correct the EGF-RvIII vector. We combined the correct 5' piece of EGF-RvIII with the 3' piece of the wild-type (wt) EGF-R (pLXSN vector supplied by Dr. Shelton Earp). A 1.4 kb *Hind*III/*Bam*HI fragment of the EGF-RvIII clone was ligated into pcDNA3.1 Zeo. One of several positive clones (clone 5)was used for all further constructions. DNA of clone 5 and also pLXSN wtEGF-R were digested with *Ppu*MI/*Pst*I. *Ppu*MI/*Pst*I cuts 212 bp downstream of the desired deletion mutation and before the first undesired mutation. The 3.3 kb *Ppu*MI/*Pst*I fragment derived from pLXSN/wtEGF-R was ligated into the cut clone 5/EGF-RvIII/pcDNA3. The corrected EGF-RvIII vector was sequenced and the sequence was found to be 100% identical with the published sequence in gene bank.

During the past year (no cost extension period) the corrected EGF-RvIII vector was used to transfect rat mammary carcinoma cells MTLN3. Six independent transfections were performed using lipofectamine and FuGene methods. Transfectants were selected by FACS analyses using anti-EGF-RvIII MAb L8. However, there was no enrichment for antigen-positive cells (<5% cells positive after sorting). In contrast, we have successfully transfected a rat glioma cell line. These transfectants are >80% positive for EGF-RvIII. Thus, the vector is functional, but the rat mammary cells MTLN3 do not stably integrate the vector. We will now try to transfect another rat mammary carcinoma cell line (MTCT10).

Task 2: Production and characterization of peptides of rat EGF-RvIII and corresponding control peptides.

Five different rat EGF-RvIII peptides were selected for expression of MHC class I and/or class II binding motives. Only one peptide (peptide 4) bound specifically to MAb L8 (see Table 1 of the July 1997 report and Fig. 1 of the July 1998 report for details).

Task 3: Production and characterization of rat EGF-RvIII derived from recombinant baculovirus.

A baculovirus expression vector for rat EGF-RvIII was constructed and the presence of the correct insert was confirmed by sequencing. Recombinant baculovirus was produced and used to infect insect cells. The supernatant of the infected insect cells was tested by Western blot analysis for the presence of the mutated protein (extracellular domain), using MAb NCL-EGF-R to the extracellular domain of human wt EGF-R as a probe. MAb NCL-EGF-R reacted with the 41 kDa band characteristic of the extracellular domain of rat EGF-RvIII in 24 hr supernatants. The protein was purified from the supernatant by HPLC and tested by Western blot analysis and ELISA using MAb L8. The characteristic 41 kDa band of EGF-RvIII was detected by Western blot analysis and MAb L8 specifically reacted with EGF-RvIII protein in ELISA.

Rats were immunized with rat EGF-RvIII protein in complete and incomplete Freund's adjuvant. There was a trend for the lower antigen dose yielding higher antibody response than the higher dose. The antibodies not only bound to rat EGF-RvIII, but also to normal rat EGF-R protein. Thus, rats are not immunologically tolerant to normal EGF-R administered in adjuvant although this protein is widely expressed by normal rat tissues. Immune responses to normal EGF-R were not accompanied by toxicity as determined macroscopically in those organs which express EGF-R. These data have been described in detail in Figs. 4, 5, and 6 of the July 1997 report and Figs. 2 and 3 of the July 1998 report.

Task 4: In vitro responses of rat lymphocytes to stimulation with peptides.

These studies could not be performed because we lack syngeneic rat mammary carcinoma cells expressing the EGF-RvIII epitope. These cells are needed to test proliferating lymphocytes from EGF-RvIII-immunized mice for cytolytic activity.

Task 5: Production and characterization of six peptides of human EGF-RvIII and corresponding control peptides.

We have produced six peptides of human EGF-RvIII which were described in detail in the previous reports. The peptides were selected for expression of anchors for HLA class I and/or II (for details see Table in the 1998 report).

Task 6: Immunization of rats with rat EGF-RvIII peptides and characterization of immune responses.

Peptides of rat EGF-RvIII were incorporated into microspheres and injected with or without Titermax adjuvant. Of the five peptides, only peptide 4 (with or without adjuvant) and peptide 5 (with adjuvant only) induced antibodies binding specifically to EGF-RvIII as compared to bovine serum albumin. No such antibodies were induced in the control-immunized rats. These data have been described in detail in Fig. 3 of the 1998 report.

Task 7: Tumor protection studies with peptides in rats.

These studies could not be performed because we lack syngeneic rat mammary carcinoma cells expressing the EGF-RvIII epitope.

Task 8: Production and in vitro characterization of up to 20 anti-idiotypes.

We will receive from Dr. Darell Bigner two anti-idiotypic antibodies directed against monoclonal anti-EGF-RvIII antibodies Y10. These anti-idiotypes mimic EGF-RvIII in vitro and induce tumor protective immune responses in a mouse tumor model (D. Bigner, personal communication). The anti-idiotypes will be tested in our rat model of EGF-RvIII when the target cells become available. The murine anti-idiotypes most likely will be more immunogenic in xenogeneic rats than in syngeneic mice (our unpublished observations) and, therefore, they may have superior anti-tumor activity in our rat model as compared to Dr. Bigner's mouse model.

Task 9: In vivo characterization of 2-3 selected anti-idiotypes.

These studies will be performed when syngeneic EGF-RvIII expressing mammary carcinoma cells will be available.

Task 10: In vitro stimulation of patients' lymphocytes with peptides and anti-idiotypes.

We have produced peptides of human EGF-RvIII (see task 5) and recombinant human EGF-RvIII protein for the stimulation of cancer patients' lymphocytes in vitro. Positive lymphocyte stimulation would indicate that the peptides and/or the protein may serve as vaccines for breast cancer patients. Human EGF-RvIII extracellular domain was produced in recombinant baculovirus. Western blot analysis of purified human EGF-RvIII protein derived from baculovirus-infected insect cells revealed the 30 kDa protein characteristic of the extracellular domain of human EGF-RvIII. Analysis of the purified protein in ELISA shows its reactivity with both MAb NCL-EGF-R to human wtEGF-R and MAb L8 to human EGF-RvIII. These data have been described in detail, supported by Figs. 5 and 6, in the July 1997 report.

Preliminary studies shown in the previous report (July 1999) have suggested that breast cancer patients with EGF-RvIII-positive lesions produce specific antibodies against EGF-RvIII and show lymphoproliferative responses specific for EGF-RvIII. There seemed to be a correlation between the expression of EGF-RvIII by tumor tissue and humoral immune response development. However, these studies included only five patients. During the fourth year of funding an additional eleven patients were included in these studies. Figure 1 (A-G) shows

EGF-RvIII specific antibody responses determined in the sera of 7 breast cancer patients. The details of the assay used are described in Fig. 1 legend. The antibodies which bound to EGF-RvIII did not bind to wt EGF-R or carcinoembryonic antigen (CEA). In positive control experiments, antibodies to wtEGF-R or CEA bound to their respective antigen (Fig. 1H). The sera of nine additional breast carcinoma patients did not bind to EGF-RvIII (Fig. 2 A-I), neither did the sera of three healthy donors (Fig. 3). *During the past year we have tested serum reactivity of the patients against human EGF-RvIII positive transfectants NR6M and found that in 5 of the 7 patients who had developed EGF-RvIII protein-reactive antibodies these antibodies also reacted with EGF-RvIII positive cells (Table).*

We also tested lymphoproliferative responses of the above described breast cancer patients after stimulation of the lymphocytes with EGF-RvIII protein, EGF-RvIII peptides or control preparations. The details of the assay used are described in Fig. 4 legend. As shown in Fig. 4, three patients had significant lymphoproliferative responses to stimulation with EGF-RvIII protein compared to stimulation with wtEGF-R or no stimulation. Two of the three patients (DM and TF; Fig. 4 B and C) also had specific and significant lymphoproliferative responses to stimulation with EGF-RvIII peptide in PLG microspheres. In contrast, 13 other breast cancer patients did not show EGF-RvIII-specific lymphoproliferative responses, although their lymphocytes responded to phytohemagglutinin (PHA) stimulation (Fig. 5). Although we had originally planned to stimulate patients' lymphocytes with anti-idiotypes mimicking EGF-RvIIII, these studies were not performed because $F(ab')_2$ fragments of the anti-idiotype are needed for lymphocyte stimulation. These fragments were not available in sufficient quantities. Instead, we used recombinant baculovirus-derived EGF-RvIII protein for lymphocyte stimulation (see Figs. 4 and 5).

To determine whether there was a correlation between immune response to EGF-RvIII and EGF-RvIII expression by the patients' tumors, tumors were evaluated for EGF-RvIII expression by reverse transcriptase polymerase chain reaction (RT-PCR) and immunohistochemistry with MAb L8 (Table). *RT-PCR determinations were completed in all patients' tissues (except for patient EJ from whom no more tissue sample was available) during the past year. Nine of 15 patients' tissues were positive for EGF-RvIII by RT-PCR. Four of these patients (DI, KJ, MC, HrJ) had developed antibodies binding specifically to the mutated protein.*

All five patients with lesions positive for EGF-RvIII by immunohistochemistry (KJ, MC, BD, EJ, KrJ) had developed specific antibodies to EGF-RvIII baculovirus-derived protein (Table). One patient (TF) had humoral and cellular immune responses, but EGF-RvIII-negative lesions as determined both by RT-PCR (see above) and immunohistochemistry. The specimen obtained from this patient's lesion may not be representative of the tumor as a whole.

Only three patients (KJ, TF, DM) had EGF-RvIII-specific lymphoproliferative responses. These responses correlated with tumor EGF-RvIII expression in only one patient (KJ).

There was a significant correlation ($p=0.03$; Fisher's exact test) between patients' humoral immune responses to EGF-RvIII protein and EGF-RvIII tumor expression (determined

by immunohistochemistry. There was no significant correlation between cellular immune responses and EGF-RvIII expression by immunohistochemistry or either cellular or humoral immune responses and EGF-RvIII detection by RT-PCR. Absence of a correlation between immune response and EGF-RvIII presence by RT-PCR is not surprising in the absence of protein expression (patients PL, RS, BR, DM). Immunohistochemistry and RT-PCR data also did not significantly correlate in the group of patients, presumably because of sensitivity differences between the two assays or different parts of the specimens used in the two assays which are not representative of the tumor as a whole. Immune responses to EGF-RvIII protein also did not correlate with the HER2-neu or estrogen receptor status of the tumors. A larger study is needed to establish such correlation.

KEY RESEARCH ACCOMPLISHMENTS (4-year funding period and 1 year no cost extension)

- Rat mammary carcinoma cells were transfected with rat EGF-RvIII, but expression of the mutated epitope was instable, probably due to additional mutations in the rat EGF-RvIII vector. The vector has been corrected, *but was unable to transfer EGF-RvIII expression to rat mammary carcinoma cells.*

- Various human and rat-derived EGF-RvIII peptides, recombinant proteins, and EGF-RvIII expressed in adenovirus have been produced. These reagents serve as targets or stimulants for the determination of humoral or cellular immune responses in rats and humans.

- Rats produced antibodies to EGF-RvIII peptides and baculovirus-derived protein. Immune responses were directed both to wt and mutated sequences of EGF-RvIII. Thus rats are not immunologically tolerant to wtEGF-R despite the expression of this protein by many normal rat tissues.

- Breast cancer patients develop antibodies and proliferative lymphocyte responses to EGF-RvIII expressed by their tumors. These responses are specific for the mutated epitope on EGF-RvIII. *Antibody responses to EGF-RvIII significantly correlated with EGF-RvIII protein expression by the patients' tumors.*

REPORTABLE OUTCOMES

Abstracts (see appendix)

Immune responses of breast cancer patients to mutated epidermal growth factor receptor (mEGF-R); Purev, E., Cai, D.W., Miller, E., Birebent, B., Somasundaram, R., Mayer, T., and Herlyn, D.; Albert Einstein Med. Ctr., Philadelphia, PA, Memorial Hosp. of Burlington, Mt. Holly, NJ, and The Wistar Institute, Philadelphia, PA. Scientific Proceedings, 91st Annual Meeting of the American Association for Cancer Research, April 1-5, 2000, San Francisco, CA.

Immune responses of breast cancer patients to mutated epidermal growth factor receptor (mEGF-R); Purev, E., Cai, D.W., Miller, E., Birebent, B., Somasundaram, R., Mayer, T., and Herlyn, D.; The Wistar Institute, Philadelphia, PA 19104, Memorial Hospital of Burlington, Mt. Holly, NJ 08060, and Albert Einstein Medical Center, Philadelphia, PA 19104. Era of Hope Meeting, June 8-11, 2000, Atlanta, GA.

Development of tissue and serum repositories.

Tumor tissue, lymphocyte, and serum repositories have been developed from sixteen breast cancer patients, most of them with primary tumors without lymph node involvement.

Funding applied for.

We have applied to the National Cancer Institute for funding of a study on immune responses of breast cancer patients to EGF-RvIII and the prognostic value of EGF-RvIII for clinical outcome. The revised application will be submitted 11-1-01.

CONCLUSIONS

We have developed a rat model of active specific immunotherapy against mammary carcinoma targeting EGF-RvIII. EGF-RvIII is a tumor-specific antigen which is not expressed by any normal tissues tested. During the funding period, various vaccines of rat and human EGF-RvIII have been developed. These include peptides, recombinant proteins and the protein expressed in adenovirus which will be tested for their tumor growth inhibiting properties in vivo when rat mammary carcinoma cells expressing the EGF-RvIII epitope become available. Although we performed numerous transfections of rat mammary carcinoma cells with rat EGF-RvIII cDNA, the transfectants expressed the mutated protein but not the mutated epitope. Our future studies are aimed at generating transfectants which express the mutated epitope and use them in studies of cellular and protective immunity against EGF-RvIII.

We have shown that breast cancer patients raise specific immune responses to EGF-RvIII. These responses are expected to be induced by EGF-RvIII expressed by the patients' tumors, and there was a statistically significant correlation between the humoral immune response to EGF-RvIII protein and EGF-RvIII expression by the patient's tumor. Future studies will determine whether EGF-RvIII expression by the patients' tumors is a prognostic factor which determines clinical outcome of the disease in breast cancer patients.

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APPENDICES

Abstracts, Table, Figures 1-5.

SCIENTIFIC PROCEEDINGS

91st Annual Meeting of the American Association for Cancer Research

San Francisco, CA
April 1–5, 2000

#4443 IMMUNE RESPONSES OF BREAST CANCER PATIENTS TO MUTATED EPIDERMAL GROWTH FACTOR RECEPTOR (MEGF-R). Enkhtsetseg Purev, D. W Cai, E. Miller, B. Birebent, R. Somasundaram, T. Mayer, and D. Herlyn, *Albert Einstein Med Ctr, Philadelphia, PA, Memorial Hosp of Burlington, Mt. Holly, NJ, and The Wistar Institute, Philadelphia, PA*

mEGF-R is expressed by carcinomas of the breast, ovary, lung and colon, and also by gliomas, but not by normal tissues. The mEGF-R is the result of an 801 bp deletion within the extracellular domain of normal EGF-R and is expressed both on the surface and in the cytoplasm of the tumor cells. Thus, mEGF-R is a potential tumor-specific target for B and/or T cells in active and passive immunotherapy against tumors. We have evaluated humoral and cellular immune responses to mEGF-R in eight breast cancer patients and three healthy donors. Four patients with tumors expressing mEGF-R developed mEGF-R-specific humoral immune responses and two of these patients also developed mEGF-R-specific cellular immune responses. None of the patients with mEGF-R negative tumors developed cellular immune responses, and only one patient developed antibodies to mEGF-R. None of the three healthy donors demonstrated mEGF-R-specific humoral or cellular immune responses. These studies demonstrate that breast cancer patients can immunologically recognize mEGF-R and suggest that enhancement of the immune responses may be possible by vaccination of the patients against mEGF-R. (Supported by DAMD17-96-1-62 37 from the U.S. Department of Defense.)

Era of Hope Meeting
June 8-11, 2000
Atlanta, GA

IMMUNE RESPONSES OF BREAST CANCER PATIENTS TO MUTATED EPIDERMAL GROWTH FACTOR RECEPTOR (MEGF-R)

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Epidermal growth factor binds to epidermal growth factor receptor (EGF-R) located on normal cells. The binding initiates cell division and growth. When cells become cancerous, EGF-R is mutated (mEGF-R) due to the deletion of 261 amino acids in the extracellular domain. MEGF-R expression is directly correlated with the degree of cancer malignancy. Several cancers including carcinomas of breast, ovary, lung and gliomas express mEGF-R. Normal cells do not express mEGF-R. Thus, mEGF-R is a potential target for active specific immunotherapy against cancer. We have evaluated the cellular and humoral immune responses to mEGF-R in eight breast cancer patients and three healthy donors. Four patients with tumors expressing mEGF-R developed antibodies binding specifically to mEGF-R, but not to normal EGF-R. Only one of the four other patients with tumors that lacked mEGF-R expression developed antibodies to mEGF-R. None of the three healthy donors demonstrated mEGF-R specific antibodies. Two of eight patients developed lymphoproliferative responses to stimulation with mEGF-R protein, but not with control proteins. Both patients' tumors expressed mEGF-R. None of the three healthy donors demonstrated such lymphoproliferative responses. This study demonstrates that breast cancer patients can immunologically recognize mEGF-R and suggests that enhancement of the immune responses may be possible by vaccination of cancer patients against mEGF-R. (Supported by DAMD17-96-1-62 37 from the U.S. Department of Defense).

Table. Summary of patients' clinical status and immune responses

Patients		Tumor				Time interval between surgery and blood sampling (months)	Tumor[a] expression of EGF-RvIII		Immune responses to EGF-RvIII		
									Humoral		
Initials (Age in years)	HLA-type	Lymph node involvement	Diameter (cm)	Estrogen Receptor	HER2-neu		RT-PCR	Immunohistochemistry	Protein	Cells	Cellular
DI (74)	A*02; B*07,1401; Cw*07,1505; DRb1*0101,11; DQb1* 0301,0501; DRb3*02	+	5.0	+	NT	2.5	+	-	+	+	-
KJ (51)	A*01; B*57; Cw*06; DRb1*0701,11; DQb1* 0301,0303; DRb3*02; DRb4*01	-	2.0	+	NT	37	+	+	+	+	+
MC (71)	A*01,03; B*15,57; Cw*03,06; DRb1*1501,11; DQb1* 0301,06; DRb3*02; DRb5*01	-	1.4	+	NT	2.5	+	+	+	+	-
TF (62)	A*01,03; B*07; Cw*07; DRb1*0101,1501; DQb1* 0501,06; DRb5*01	-	4.5	+	NT	44	-	-	+	+	+
CR (45)	A*02,03; B*07,44; Cw*0501,0702; DRb1*1501,14; DQb1* 0503,06; DRb3*02; DRb5*01	-	1.5	NT	NT	38	-	-	-	-	-
FM (36)	A*0201; B*27,40; Cw*0102,0304; DRb1*1501,0801; DQb1* 04,06; DRb5*01	+[b]	2.5	-	NT	44	-	-	-	-	-
GI (70)	A*01,24; B*07,08; Cw*07,0702; DRb1*03,04; DQb1*02,03; DRb3*01; DRb4*01	+	5.0	NT	NT	45	-	-	-	-	-
BD (48)	A*01,03; B*07,08; Cw*07; DRb1*03, 0402; DQb1*02, 03;DRb 3*01,4*01	+	2.0	-	-	before surgery	+	+/-	+	-	-
PL (42)	A*01,02; B*08; Cw*0701; DRb1*03,15; DQb1*02,06;DRb 3*01, 5*01	-	2.0	+	-	before surgery	+	-	-	-	-
RS (66)	A*02,09; B*44; Cw*05,1601; DRb1*04,04; DQb1*03,03;DRb4*01	-	2.1	NT	+	before surgery	+	-	-	-	-

Table. Summary of patients' clinical status and immune responses (continued)

Patients			Tumor				Time interval between surgery and blood sampling (months)	Tumor[a] expression of EGF-RvIII		Immune responses to EGF-RvIII		
										Humoral		
Initials	(Age in years)	HLA-type	Lymph node involvement	Diameter (cm)	Estrogen Receptor	HER2-neu		RT-PCR	Immunohistochemistry	Protein	Cells	Cellular
BR	(61)	A*01,6601; B*5802; Cw*0602,1801; DRb1*13,1602; DQb1*0303,05;DRb 3*02, 5*02	-	1.2	+	+	before surgery	+	-	-	-	-
DM	(66)	A*02,3402; B*35,53; Cw*0401,0602; DRb1*03,1503; DQb1*02,0506;DRb 3*02, 5*00	-	1.1	NT	+	before surgery	+	-	-	-	+
DP	(38)	A*01; B*08; Cw*0701; DRb1*03,15; DQb1*02,06;DRb 3*01, 5*01	+	1.4	-	+	before surgery	-	-	-	-	-
GB	(52)	A*0201,02,32; B*07,35; Cw*04,0702; DRb1*08,11; DQb1*03,04;DRb 3*02	-	1.5	NT	+	before surgery	-	-	-	-	-
EJ	(59)	A*0202,02,03; B*15,41	-	1.9	+	-	before surgery	NT	+/-	+	+	-
KrJ	(71)	A*01,2501; B*18,44; DRb1*14,15; DQb1*0503,06;DRb 3*02, 5*01	-	4	-	NT	before surgery	-	+	+	-	-

[a] Primary tumor.

[b] Expression of EGF-RvIII was tested on the primary breast cancer lesion.

NT not tested

FIGURE LEGENDS

Fig. 1. Antibodies to EGF-RvIII in sera of breast cancer patients. Sera from breast cancer patients (panel A-G; for EGF-RvIII expression by the lesions, see table) were tested for binding to EGF-RvIII by measuring their capacity to inhibit binding of ^{125}I-labeled anti-EGF-RvIII MAb L8A4 to EGF-RvIII in radioimmunoassay. Wells coated with 10 μg/ml of anti-wtEGF-R MAb 425 were incubated first with 10 μg/ml of purified EGF-RvIII protein and then with serum dilutions (or 1-20 μg/ml of positive control MAb L8A4; panel H). ^{125}I-labeled MAb L8A4 (20 K cpm/well) was added and % inhibition of binding of ^{125}I-MAb L8A4 to EGF-RvIII by sera or MAb L8A4 was calculated relative to buffer control.

To measure binding of the sera to wtEGF-R, wells coated with MAb 425 were incubated with 10 μg/ml of purified wtEGF-R protein followed by the addition of serum dilutions (panels A-G) (or 1-20 μg/ml positive control MAb NCL directed to wtEGF-R, panel H) and 20K cpm per well of ^{125}I-labeled MAb NCL.

To measure binding of the sera to CEA, wells were coated with 10 μg/ml of anti-CEA MAb followed by incubation with 10 μg/ml of CEA, serum dilutions (panels A-G) (or 1-20 μg/ml of polyclonal antibody [PAb] to CEA, panel H) and 20K cpm per well of ^{125}I-labeled anti-CEA PAb. *, values obtained in the EGF-RvIII system are significantly (at $p < 0.05$ level; two-sided student's t-test) different from the control values obtained in the wtEGF-R or CEA systems.

Fig. 2. Absence of binding of breast cancer patients' sera to EGF-RvIII. Sera from breast cancer patients (panels A-I) with EGF-RvIII$^-$ lesions were tested for binding to EGF-RvIII, wtEGF-R or CEA as described in Fig. 1 legend. As positive control inhibitors MAb L8A4, MAb NCL and polyclonal antibody (PAb) to CEA were used as described in Fig. 1 legend. *, values obtained with MAb and PAb are significantly (at $p < 0.05$ level; two-sided student's t-test) different from the buffer control values.

Fig. 3. Absence of healthy donors' sera binding to EGF-RvIII. Sera from healthy donors (panels A-C) were tested for binding to EGF-RvIII, wtEGF-R or CEA as described in Fig. 1 legend. As positive control inhibitors MAb L8A4, MAb NCL and polyclonal antibody (PAb) to CEA were used as described in Fig. 1 legend. *, values obtained with MAb and PAb are significantly (at $p < 0.05$ level; two-sided student's t-test) different from the buffer control values.

Fig. 4. Proliferation of breast cancer patients' PBMC to stimulation with EGF-RvIII protein. For expression of EGF-RvIII by patients' lesions see Table. PBMC were stimulated for 10 days (TF) or 5 days (KJ, DM) with 10 μg/ml of EGF-RvIII or wtEGF-R protein, 25 μg/ml of EGF-RvIII peptide (patient DM: peptide GAALLALLAALCPASR ALEEKKGNY; patients KJ, TF: peptide ALEEKKGNY), in the presence of 1 μg/ml of β2 microglobulin, the EGF-RvIII peptides in PLG microspheres, or control (co) peptide GEXXLAEKLN. PHA was used as a positive control. PBMC were then pulsed with ^{3}H-thymidine and ^{3}H-thymidine incorporation was measured. Values with identical letters differ significantly from each other in two-sided student's t-test.

Fig. 5. Absence of lymphoproliferative responses to EGF-RvIII stimulation in breast cancer patients. For EGF-RvIII expression by patients' lesions see Table. PBMC were stimulated for 10 days (GI) or 5 days (DI, MC, CR, FM, KrJ, BD, PL, RS, BR, DP, GB, EJ) with EGF-RvIII or wtEGF-R protein, EGF-RvIII peptide (patients DI, FM, RS, GB: peptide GAALLALLAALCPASR ALEEKKGNY; patients MC, CR, KrJ, GI, BD, PL, BR, DP, EJ: peptide ALEEKKGNY), control (co) peptide GEXXLAEKLN or PHA as described in Fig. 4 legend. PBMC were then pulsed with ^{3}H-thymidine and ^{3}H-thymidine incorporation was measured. Values with identical letters differ significantly from each other in two-sided student's t-test.

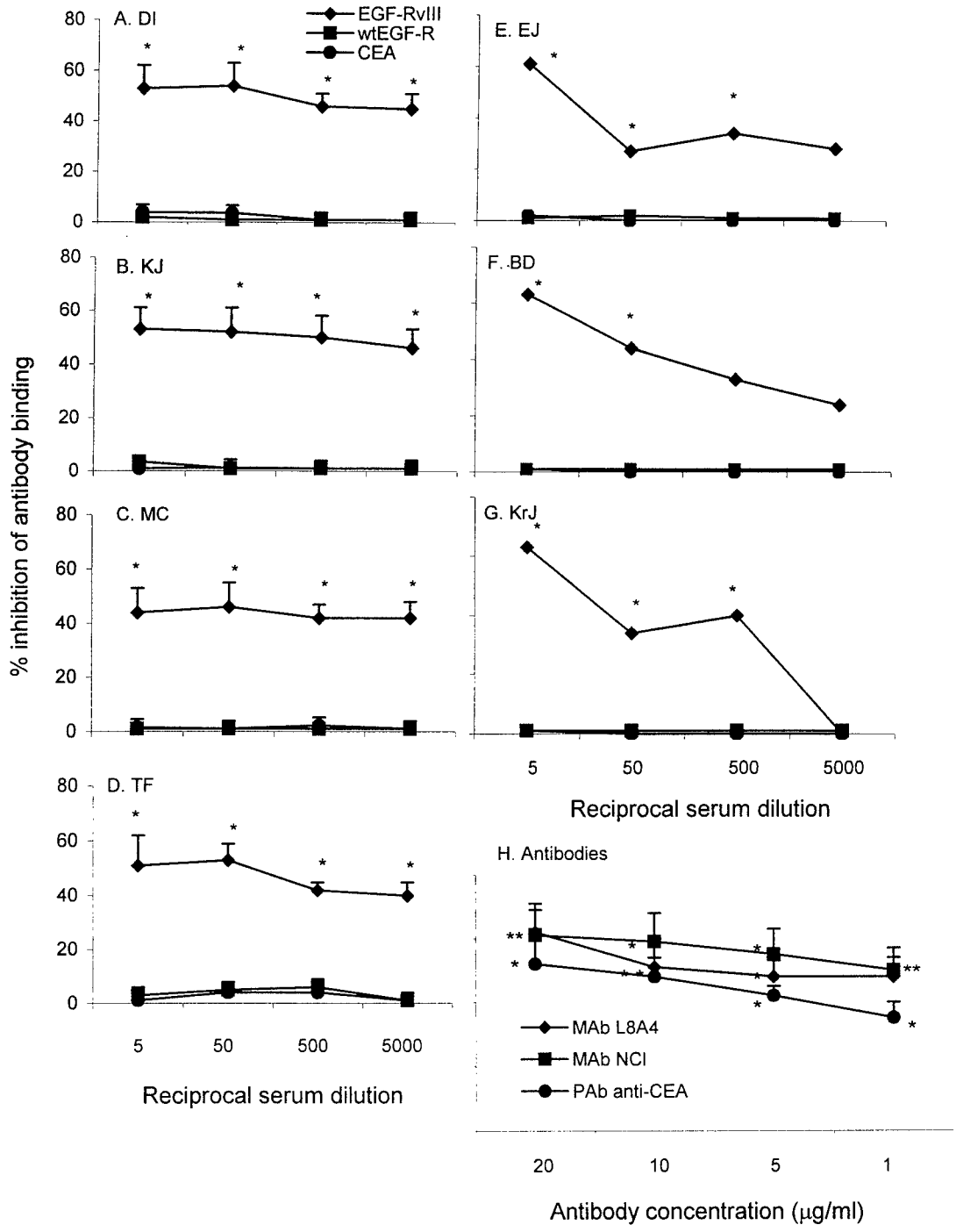
A. DI
EGF-RvIII
wtEGF-R
CEA
E. EJ
B. KJ
F. BD
C. MC
G. KrJ
D. TF
H. Antibodies
% inhibition of antibody binding
80
60
40
20
0
5
50
500
5000
Reciprocal serum dilution
MAb L8A4
MAb NCI
PAb anti-CEA
20
10
5
1
Antibody concentration (μg/ml)

Figure 1

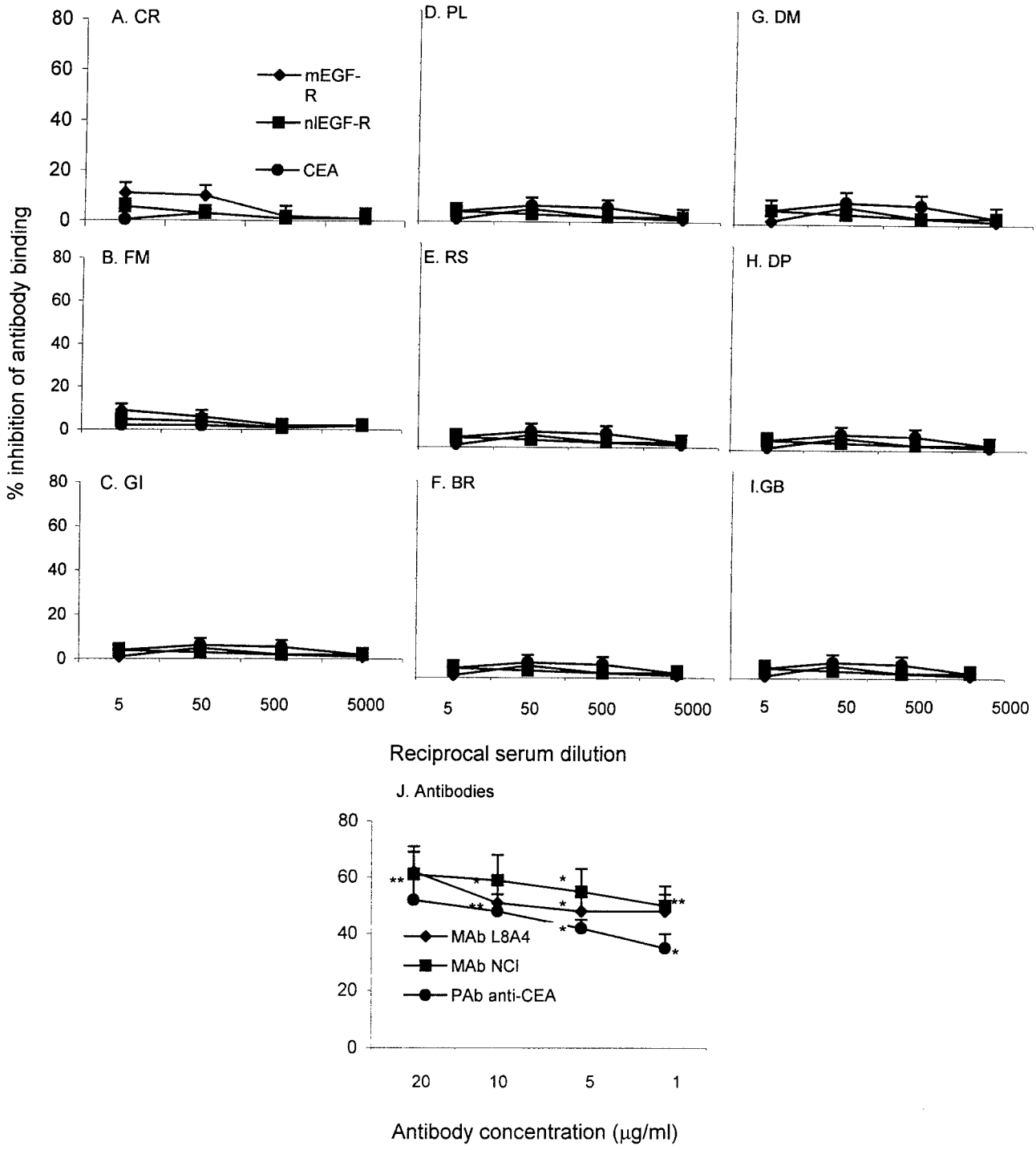
A. CR
D. PL
G. DM
B. FM
E. RS
H. DP
C. GI
F. BR
I.GB
mEGF-R
nlEGF-R
CEA
% inhibition of antibody binding
Reciprocal serum dilution
J. Antibodies
MAb L8A4
MAb NCI
PAb anti-CEA
Antibody concentration (μg/ml)

Figure 2

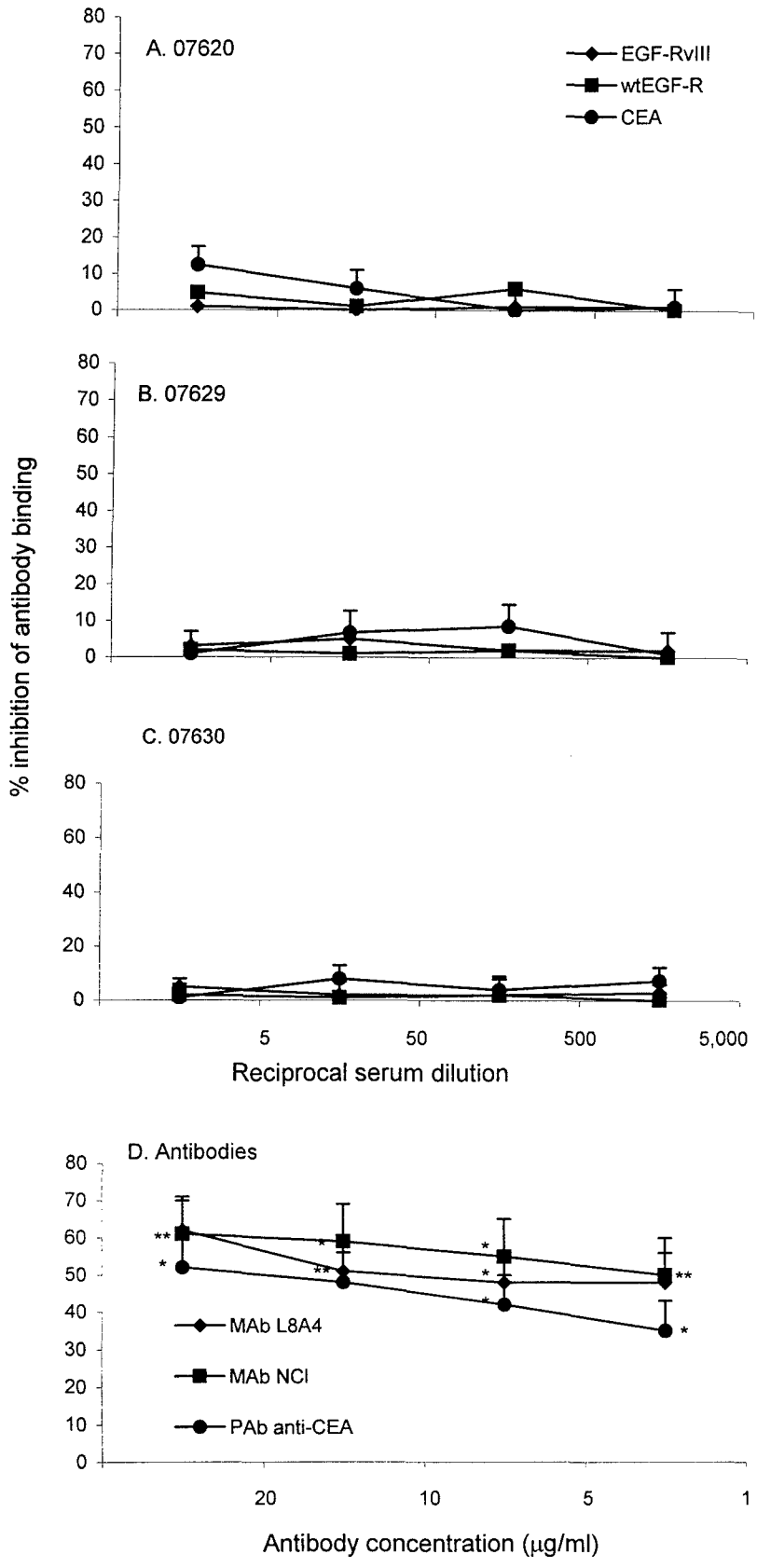
A. 07620
EGF-RvIII
wtEGF-R
CEA
B. 07629
C. 07630
% inhibition of antibody binding
Reciprocal serum dilution
D. Antibodies
MAb L8A4
MAb NCI
PAb anti-CEA
Antibody concentration (μg/ml)

Figure 3

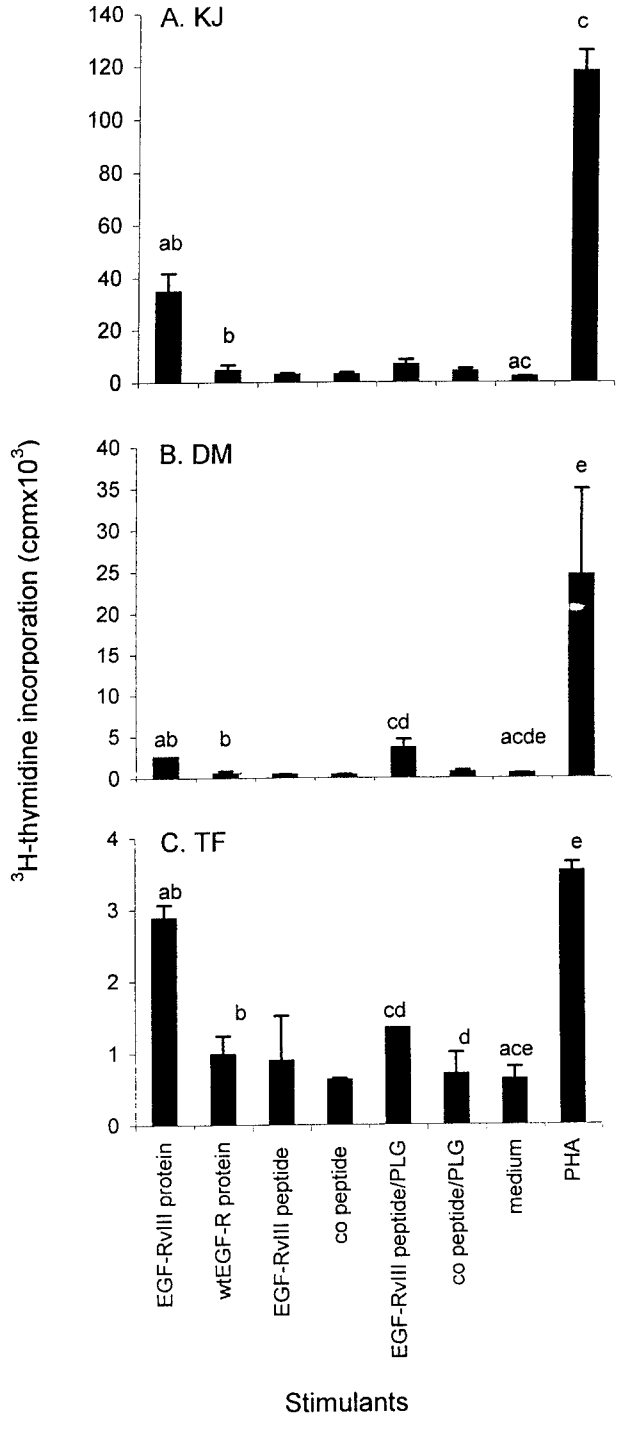
A. KJ
B. DM
C. TF
^{3}H-thymidine incorporation (cpmx10^3)
Stimulants
EGF-RvIII protein
wtEGF-R protein
EGF-RvIII peptide
co peptide
EGF-RvIII peptide/PLG
co peptide/PLG
medium
PHA

Figure 4

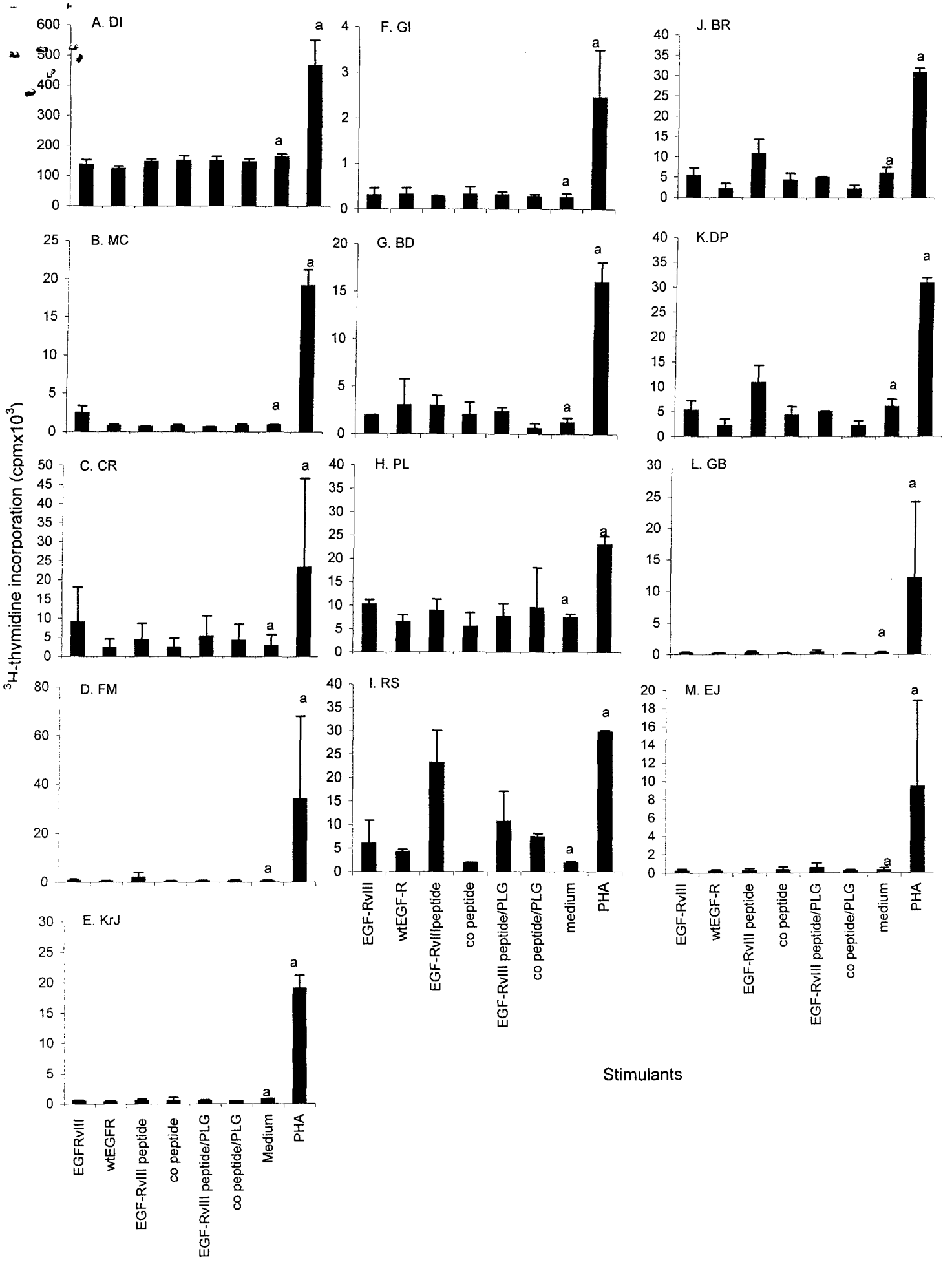
A. DI
F. GI
J. BR
B. MC
G. BD
K.DP
C. CR
H. PL
L. GB
D. FM
I. RS
M. EJ
E. KrJ
^{3}H-thymidine incorporation (cpmx10^3)
EGFRvIII
wtEGFR
EGF-RvIII peptide
co peptide
EGF-RvIII peptide/PLG
co peptide/PLG
Medium
PHA
EGF-RvIII
wtEGF-R
EGF-RvIIIpeptide
co peptide
EGF-RvIII peptide/PLG
co peptide/PLG
medium
PHA
Stimulants
Stimulants

Figure 5

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 28 Aug 02

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

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1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

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